SUMMARY OF RESEARCH

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GRANT TITLE: An Evaluation of Collagen Metabolism in Non Human Primates Associated with the Bion 11 Space Program-Markers of Urinary Collagen Turnover and Muscle Connective Tissue.

PRINCIPAL INVESTIGATOR: Arthur C. Vailas, Ph.D.
Vice Chancellor for Research and Intellectual Property Management, UH Systems, Vice President for Research, University of Houston
Co-Director, Connective Tissue Research Laboratory
Tel:713-743-9104
Fax:713-743-9125
Email: availas@uh.edu

INSTITUTION: UNIVERSITY OF HOUSTON
4800 Calhoun Blvd
Houston, Texas 77204-2162

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AN EVALUATION OF COLLAGEN METABOLISM IN NON HUMAN PRIMATES ASSOCIATED WITH THE BION 11 SPACE PROGRAM-MARKERS OF URINARY COLLAGEN TURNOVER AND MUSCLE CONNECTIVE TISSUE.

Principal Investigator: Arthur C. Vailas, Ph.D.
Vice Chancellor for Research and Intellectual Property Management, UH Systems, Vice President for Research, University of Houston
Co-Director, Connective Tissue Research Laboratory
4800 Calhoun Blvd
E. Cullen Building
University of Houston
Houston, Texas 77204-2162
Tel:713-743-9104
Fax:713-743-9125
Email: availas@uh.edu

Co-Principal Investigator: Daniel A. Martinez, Ph.D.
Research Assistant Professor, Department of Biochemistry and Biology, Division of Molecular and Cellular Biology
Co-Director, Connective Tissue Research Laboratory
University of Houston
Houston, Texas 77204-5513
Tel: 713-743-2662
Fax: 713-743-2636
Email: ddam@uh.edu

Technical Monitor: Richard E. Grindeland, Ph.D.
Chief Scientist Bion Program
NASA-Ames Research Center
Moffett Field, CA 94035-1000
Tel: 415-604-5756

NASA-Ames Grant Officer: Beatrice Morales
University Affairs Grants Office
NASA-Ames Research Center
Branch 241-1
Moffett Field, CA 94035-1000
Tel: 650-604-5811

UH-Research Admin.: Barbara DeHaven, Interim Director
Office of Sponsored Programs
Rm 316, E. Cullen Building
University of Houston
Houston, Texas 77204-2162
Tel: 713-743-9201
Fax: 713-743-9227

BION 11 FINAL REPORT

UNIVERSITY OF HOUSTON CONNECTIVE TISSUE PHYSIOLOGY LABORTORY

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INTRODUCTION

Patients exhibiting changes in connective tissue and bone metabolism also show changes in urinary by-products of tissue metabolism. Furthermore, the changes in urinary connective tissue and bone metabolites precede alterations at the tissue macromolecular level. Astronauts and Cosmonauts have also shown suggestive increases in urinary by-products of mineralized and non-mineralized tissue degradation. Thus, the idea of assessing connective tissue and bone response in spaceflight monkeys by measurement of biomarkers in urine has merit. Other investigations of bone and connective histology, cytology and chemistry in the Bion 11 monkeys will allow for further validation of the relationship of urinary biomarkers and tissue response. In future flights the non-invasive procedure of urinary analysis may be useful in early detection of changes in these tissues. *Purpose:* The purpose of this grant investigation was to evaluate mineralized and non-mineralized connective tissue responses of non-human primates to microgravity by the non-invasive analysis of urinary biomarkers. Secondly, we also wanted to assess muscle connective tissue adaptive changes in three weight-bearing skeletal muscles: the soleus, medial gastrocnemius and tibialis anterior by obtaining pre-flight and post-flight small biopsy specimens in collaboration with Dr. V. Reggie Edgerton's laboratory at the University of California at Los Angeles.

METHODS

Twenty-four hour samples were obtained intermittently before flight and after flight, before and after 1 G simulations of flight, and from vivarium monkeys. Urine volumes were measured and aliquots were frozen for subsequent analysis.

Collagen biomarker concentrations of urinary hydroxyproline (Hyp), hydroxylysylpyridinoline (HP cross-links), and lysylpyridinoline (LP cross-links) were assayed by reverse-phase high performance liquid chromatography (RP-HPLC).

Urinary creatinine (Cr) was quantitated using a colorimetric assay to measure potential muscle rhabdomyolysis and to normalize the connective tissue and bone mineral bio-marker concentrations.

Bone mineral metabolism was analyzed by assaying pre- and post-flight urinary calcium (Ca^{2+}) by atomic absorption. Urinary osteocalcin (Oc), a biomarker for bone formation was analyzed by immunoassay with a polyclonal antibody raised against bovine Oc with tracer and standards derived from purified *Macaca mulatta* bone Oc.

Skeletal muscle hydroxyproline, an index of collagen concentration, and the mature collagen cross-links (HP and LP) were measured on micro-biopsy specimens from the pre-flight and post-flight time periods from all three groups.

Statistics: The urine measurements were analyzed using a two factorial repeated measures analyses of variance (ANOVA) with the significance set at $P<0.05$.

RESULTS

Collagen Biomarkers: Summary Tables (Pre-Flight and Post-Flight) and Graphs (Pre-Post, Monthly and Daily Analyses) can be found in the appropriate sections.

Results indicate a high daily variance in the urine concentration of collagen metabolic biomarkers. The urinary collagen cross-link content in the postflight urines were significantly greater ($P<0.05$) in the Flight group compared to the Simulation and Vivarium groups indicating that more mature collagen had been degraded during the initial postflight recovery period in the Flight group. Creatinine analyses showed that in all groups urinary levels were significantly elevated (92%-111%) from preflight and the urinary content of non-reducible collagen cross-links (nM HP+LP/mM Cr) were overall greater during the postflight period (21%-35%).

Bone metabolism: Urinary Ca^{2+} measures were significantly lower (Flight –72%, Simulation –57%, Vivarium –31%) after flight versus preflight measurements ($P<0.05$). Oc analyses indicated that Flight (-43%) and Vivarium (-32%) had significantly lower Oc values postflight.

Muscle Connective Tissue: In the soleus muscle, the only significant changes observed were in the Vivarium group. The Hyp values were significantly greater post-flight versus preflight values ($P<0.05$). There was a trend towards decreased LP values post-flight compared to pre-flight values

($P<0.09$). No significant changes were found in the Flight or Simulated soleus muscle connective measurements. In the tibialis anterior, the only significant differences were found in the Flight group. Post-flight Hyp was significantly smaller in the Flight group in contrast to pre-flight measurements ($P<0.05$). However, HP and Total Cross-link measurements (HP+LP) tended to be larger post-flight compared to preflight measurements (HP: $P<0.07$) and (HP+LP: $P<0.09$) respectively. The medial gastrocnemius muscle demonstrated no significant differences in any measurements post-flight versus preflight ($P>0.05$)

SUMMARY FINDINGS

1. Biomarker profiles can be used as a *first level* non-invasive analyses to determine if connective tissue metabolism has been altered following spaceflight. Initial postflight measurements of collagen and mineral biomarkers, demonstrated in the first days following flight in the Daily Graphs, suggest an increase in mineralized and non-mineralized connective tissue turnover in the Flight group.

2. Elevated urine Hyp, HP+LP cross-link levels together with reduced urine Ca^{2+} levels may indicate new collagen secretion and Ca^{2+} storage, signifying new bone formation during postflight recovery.

3. Biomarker analyses investigating the temporal transitions of whole body collagen and mineral metabolism would be greatly enhanced if future experiments facilitated daily in-flight sample collection of urine from the primates aboard the unmanned space habitat.

4. Overall, no dramatic muscle connective tissue changes were observed post-flight in the SOL, MG or TA muscles. However, there was a significant decrease in skeletal muscle collagen in the post-flight TA biopsies in the Flight group with an accompanying increase in muscle collagen cross-

linking suggesting that the interstitial collagen is older "more mature" collagen. This findings were not observed in the SOL or MG muscle biopsies.

5. Fourteen days exposure to microgravity may not have been an adequate interval of time to view changes in skeletal muscle connective tissue protein. However, changes at the molecular level of collagen synthesis (measures of mRNA for COL 1A1, COL 3A1 and Lysyl Oxidase) may be evident when analyzed.

6. The biopsy samples received from Dr. Reggie Edgerton's laboratory were smaller in mass than what our current molecular biology methodology could quantitate. If our Laboratory receives further funding, we will attempt to optimize our mRNA assays for less than ~0.5-2.0 mg tissue wet weight.

TABLES

Pre-Flight and Post-Flight Urine BiomarkerAnalyses (Normalized to Creatinine)

Groups	Samples Collected	Hyp/Cr (µg/mMol)	HP/Cr (nMol/mMol)	LP/Cr (nMol/mMol)	Total Cross-links (nmol HP+LP/mmol Cr)
Flight (357,484) n = 2 (1 sample collected from 357 post-flight: pooled with 484 post flight)	**Pre-flight** (12-14)	19.89±2.29[a]	120.51±13.90[a]	21.01±2.86[ab]	141.53±16.58[ab]
	Post-flight (22)	17.39±2.92[a]	146.03±6.56[a]	25.45±1.52[a]	171.49±7.89[a]
Simulated (501,513,534) n = 3	**Pre-flight** (20-22)	9.66±1.02[b]	82.68±6.21[b]	15.74±2.56[bc]	98.41±7.73[c]
	Post-flight (30-36)	9.14±0.97[b]	112.48±12.76[a]	17.32±1.92[b]	133.09±14.01[ab]
Vivarium (396,447,448, 470,474,503) n = 6	**Pre-flight** (41-45)	10.11±0.59[b]	86.24±4.69[b]	11.74±0.71[c]	97.97±5.33[c]
	Post-flight (50-52)	8.77±0.39[b]	110.67±8.59[a]	16.66±1.30[b]	127.32±9.82[b]

Values represent means ± SE. Means with the same letter are *not* significantly different. A significant *P* value was set at $P<0.05$.

Pre-Flight and Post-Flight Urine BiomarkerAnalyses (Non-normalized to Creatinine)

Groups	Samples Collected	Hyp (µg/mL)	HP (nM)	LP (nM)	Creatinine (mM)
Flight (357,484) n = 2 (1 sample collected from 357 post-flight: pooled with 484 post flight)	**Pre-flight** (12-14)	58.77±8.81^{bcd}	388.48±68.98^{c}	67.79±11.83^{cd}	3.67±0.72^{b}
	Post-flight (22)	85.11±13.44^{a}	1020.0±209.29^{ab}	649.67±102.62^{ab}	7.76±1.83^{a}
Simulated (501,513,534) n = 3	**Pre-flight** (20-22)	38.27±5.78^{c}	359.25±52.81^{c}	61.29±7.86^{cd}	4.13±0.54^{b}
	Post-flight (30-36)	57.22±5.69^{bcd}	710.47±88.91^{ac}	111.56±13.96^{ac}	8.31±0.96^{a}
Vivarium (396,447,448, 470,474,503) n = 6	**Pre-flight** (41-45)	43.49±3.98^{c}	396.39±47.08^{c}	54.62±7.16^{d}	4.66±0.37^{b}
	Post-flight (50-52)	64.13±5.62^{ad}	1083.82±195.10^{b}	169.98±30.07^{b}	8.95±1.15^{a}

Values represent means ± SE. Means with the same letter are *not* significantly different. A significant *P* value was set at $P<0.05$.

Urinary Calcium and Osteocalcin

Pre-Flight and Post-Flight Analyses

Groups	Time (# of samples)	Calcium (Ca2+ µg/mg Creatinine)	Osteocalcin (ng OC/ mg Creatinine)
Flight (357,484) n = 2	**Pre-flight (n)**	49.4±13.7[a] (15)	2.44±0.45[a] (10)
	Post-flight (n)	18.9±3.9[b] (23)	1.39±0.24[b] (21)
Simulated (501,513,534) n = 3	**Pre-flight (n)**	52.5±16.2[a] (23)	1.09±0.22[a] (7)
	Post-flight (n)	22.8±4.5[b] (23)	3.25±0.93[a] (28)
Vivarium (396,447,448, 470,474,503) n = 6	**Pre-flight (n)**	37.3±6.0[a] (45)	2.60±0.86[a] (2)
	Post-flight (n)	25.9±3.3[a] (52)	1.76±0.47[a] (13)

Values represent means ± SE. Means with the same letter are *not* significantly different. A significant *P* value was set at $P<0.05$. [b]Flight Group Postflight Ca2+ ($P<0.02$) and OC ($P<0.03$) are significantly less than Preflight values. [b]Simulated Group Postflight Ca2+ ($P<0.05$) is significantly less than Preflight values.

Soleus Pre-Flight and Post-Flight Biopsies

Muscle Connective Tissue Analyses

Groups	Time	Hyp (µg/mg dry wt)	HP (mol HP/mol Collagen)	LP (mol LP/mol Collagen)	Total Cross-links (HP+LP)
Flight (357,484) n = 2	**Pre-flight**	5.34±0.26[a]	0.4361±0.154[a]	0.0727±0.283[a]	0.5088±0.182[a]
	Post-flight	10.92±3.49[a]	0.3897±0.128[a]	0.0529±0.005[a]	0.4426±0.132[a]
Simulated (501,513,534) n = 3	**Pre-flight**	6.06±1.82[a]	0.4940±0.075[a]	0.0690±0.024[a]	0.5629±0.098[a]
	Post-flight	11.76±4.30[a]	0.6709±0.292[a]	0.1099±0.051[a]	0.7809±0.343[a]
Vivarium (396,447,448, 470,474,503) n = 6	**Pre-flight**	6.57±1.93[a]	0.8439±0.428[a]	0.1302±0.057[a]	0.9741±0.485[a]
	Post-flight	11.01±2.14[b]	0.3667±0.050[a]	0.0448±0.008[b]	0.4115±0.058[a]

Values represent means ± SE. Means with the same letter are *not* significantly different.
[b]Vivarium Soleus Postflight Hyp ($P<0.04$) and LP (<0.09) values are significantly less than Preflight values.

Tibialis Anterior Pre-Flight and Post-Flight Biopsies

Muscle Connective Tissue Analyses

Groups	Time	Hyp (μg/mg dry wt)	HP (mol HP/mol Collagen)	LP (mol LP/mol Collagen)	Total Cross-links (HP+LP)
Flight (357,484) n = 2	**Pre-flight**	10.49±6.53[a]	0.2732±0.068[a]	0.0337±0.011[a]	0.3069±0.080[a]
	Post-flight	1.68±0.15[b]	0.8867±0.006[c]	0.1153±0.017[a]	1.003±0.011[c]
Simulated (501,513,534) n = 3	**Pre-flight**	4.12±0.70[a]	0.3441±0.024[a]	0.0597±0.009[a]	0.4039±0.027[a]
	Post-flight	4.33±1.29[a]	0.6101±0.227[a]	0.1025±0.040[a]	0.7126±0.268[a]
Vivarium (396,447,448, 470,474,503) n = 6	**Pre-flight**	4.95±1.38[a]	0.4374±0.178[a]	0.0858±0.040[a]	0.5232±0.217[a]
	Post-flight	6.16±0.75[a]	0.2695±0.030[a]	0.0422±0.006[a]	0.3117±0.034[a]

Values represent means ± SE. Means with the same letter are *not* significantly different. A significant *P* value was set at $P<0.05$..
[b]Soleus Postflight Hyp ($P<0.03$) is significantly less than the Preflight values. [c]Soleus Postflight HP ($P<0.07$) and HP+LP ($P<0.09$) values are significantly greater than Preflight values.

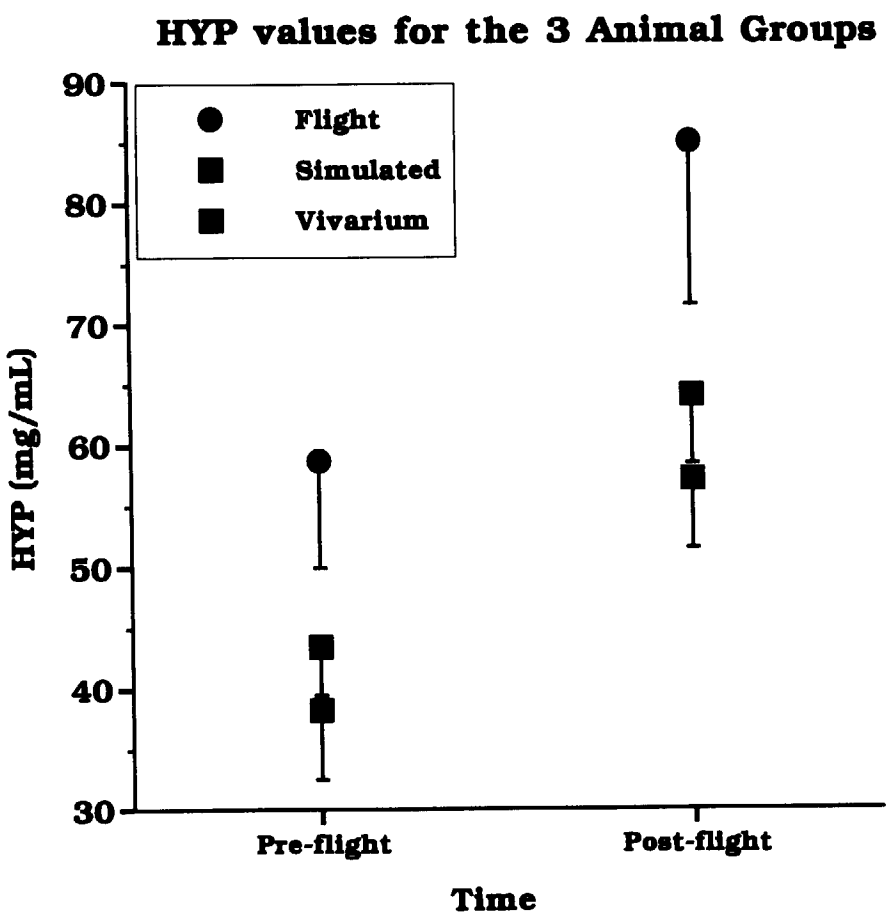
Medial Gastrocnemius Pre-Flight and Post-Flight Biopsies

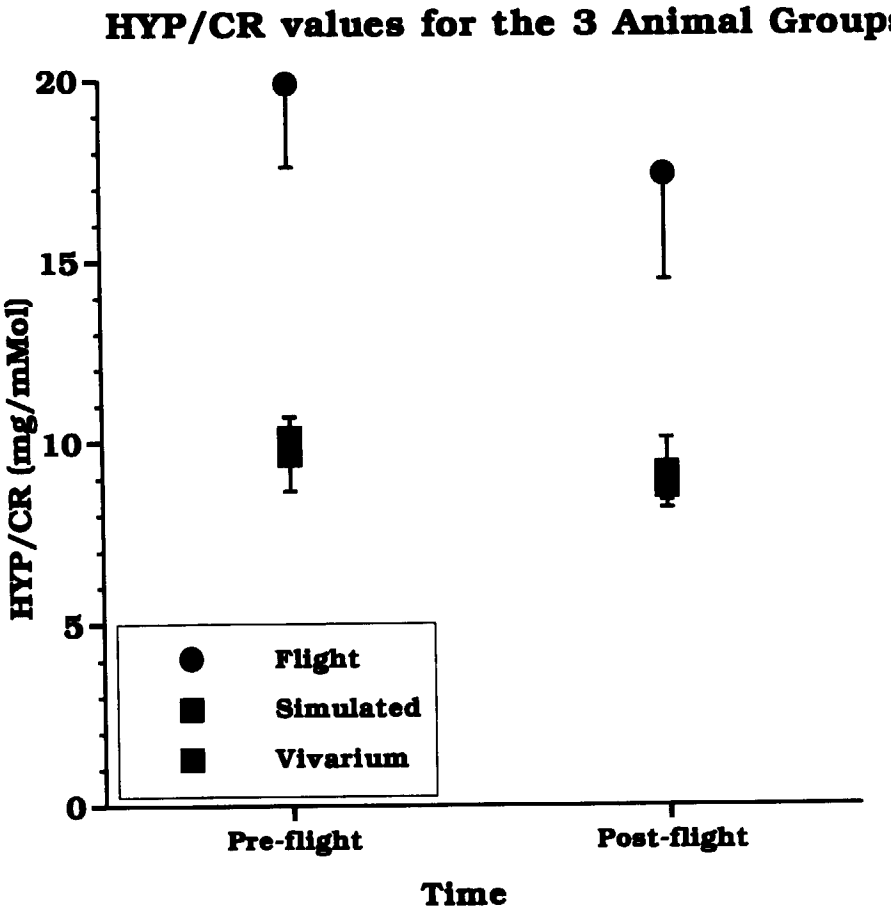
Muscle Connective Tissue Analyses

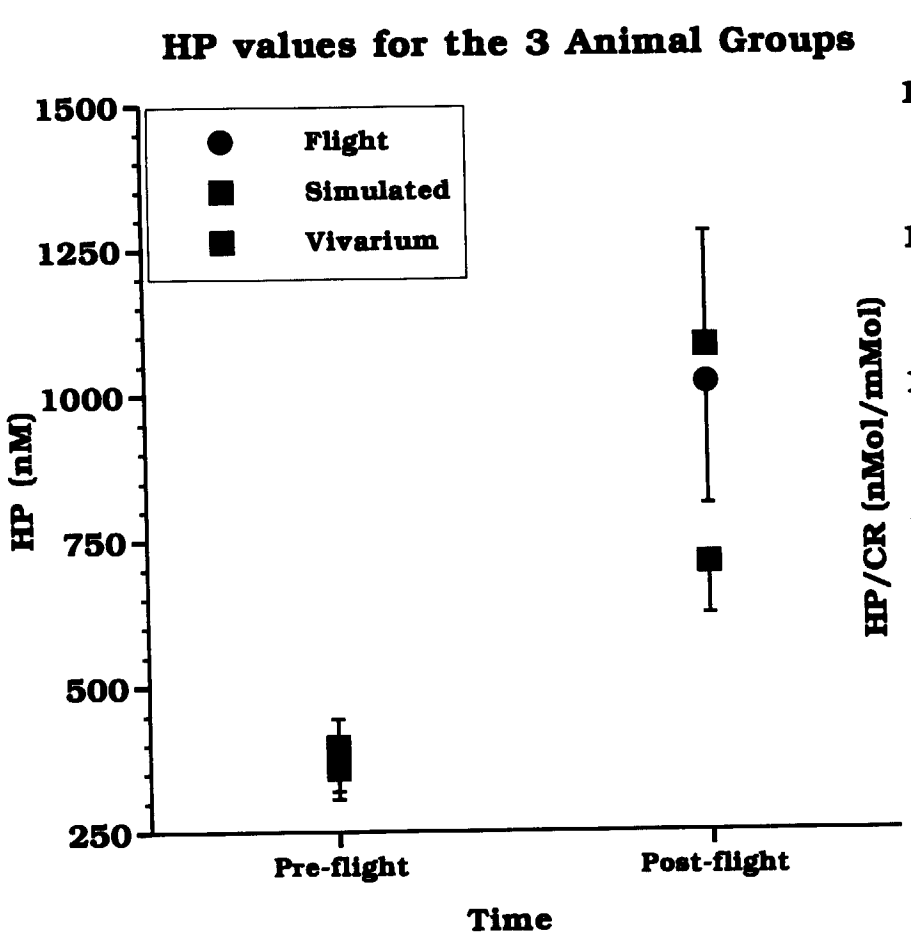
Groups	Time	Hyp (µg/mg dry wt)	HP (mol HP/mol Collagen)	LP (mol LP/mol Collagen)	Total Cross-links (HP+LP)
Flight (357,484) n = 2	**Pre-flight**	3.07±0.05	0.6561±0.120	0.0810±0.041	0.7371±0.161
	Post-flight	7.75±6.46	0.4849±0.289	0.0624±0.035	0.5473±0.324
Simulated (501,513,534) n = 3	**Pre-flight**	4.48±1.54	0.4720±0.184	0.0552±0.157	0.5272±0.199
	Post-flight	3.93±1.59	0.8010±0.347	0.1214±0.079	0.9225±0.391
Vivarium (396,447,448, 470,474,503) n = 6	**Pre-flight**	5.64±1.14	0.4774±0.172	0.0870±0.038	0.5644±0.209
	Post-flight	6.36±0.98	0.3395±0.060	0.0473±0.008	0.3868±0.067

Values represent means ± SE. Means with the same letter are *not* significantly different. There are no significant differences between Groups ($P>0.05$)

FIGURES


HYP values for the 3 Animal Groups
HYP (mg/mL)
90
80
70
60
50
40
30
Flight
Simulated
Vivarium
Pre-flight
Post-flight
Time


HYP/CR values for the 3 Animal Groups
HYP/CR (mg/mMol)
20
15
10
5
0
Flight
Simulated
Vivarium
Pre-flight
Post-flight
Time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Simulated
Vivarium
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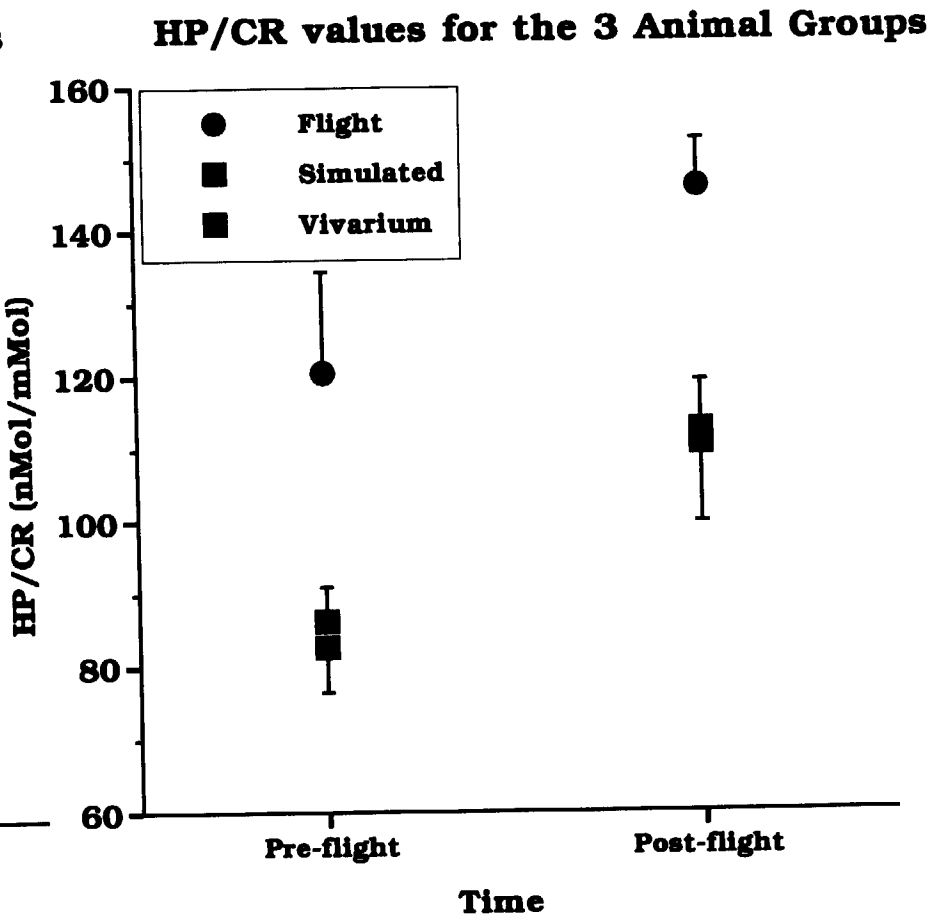
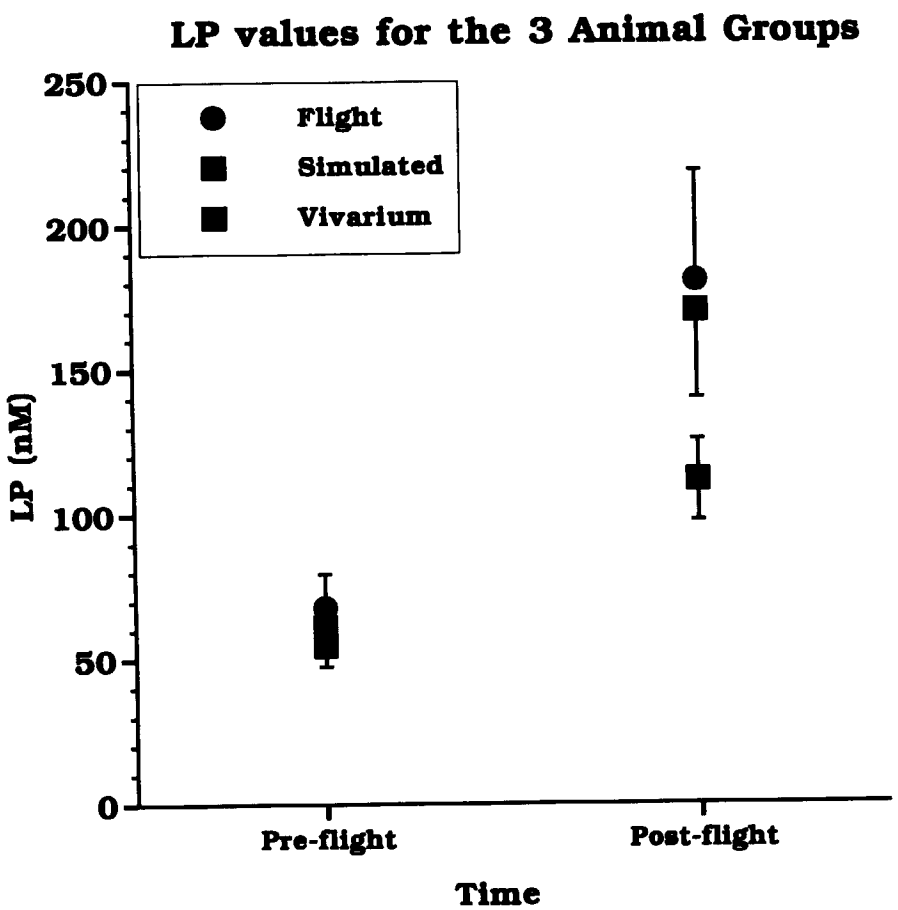
1250
1000
750
500
250
HP (nM)
Pre-flight
Post-flight
Time


HP/CR values for the 3 Animal Groups
Flight
Simulated
Vivarium
160
140
120
100
80
60
HP/CR (nMol/mMol)
Pre-flight
Post-flight
Time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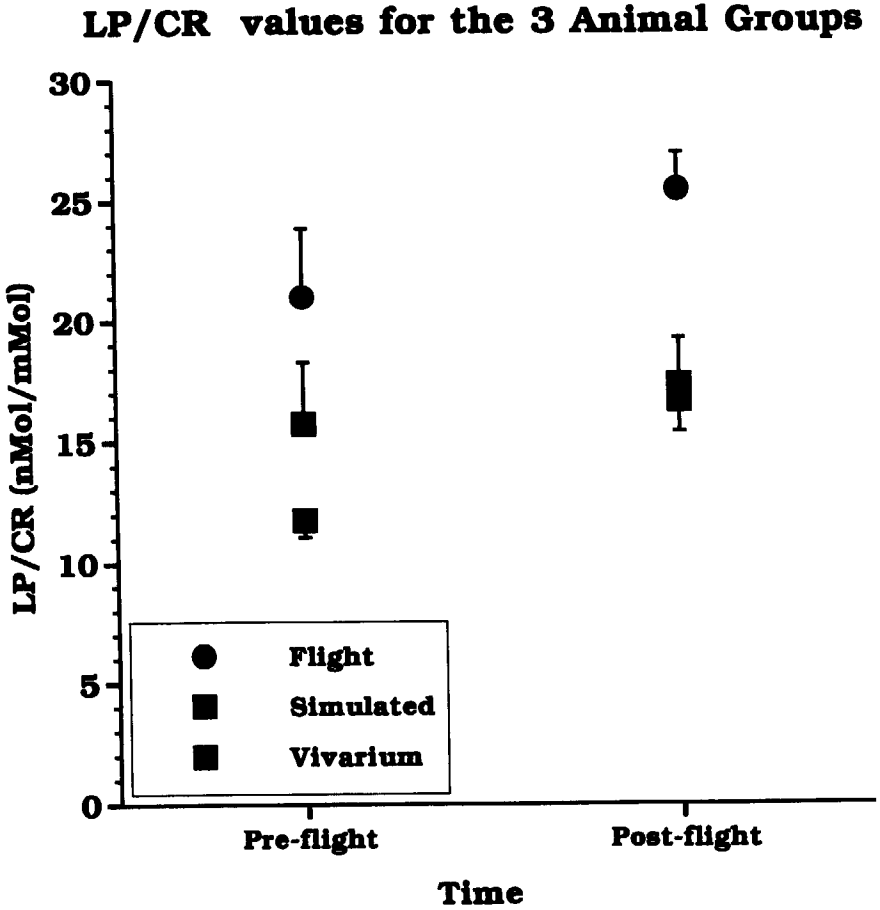
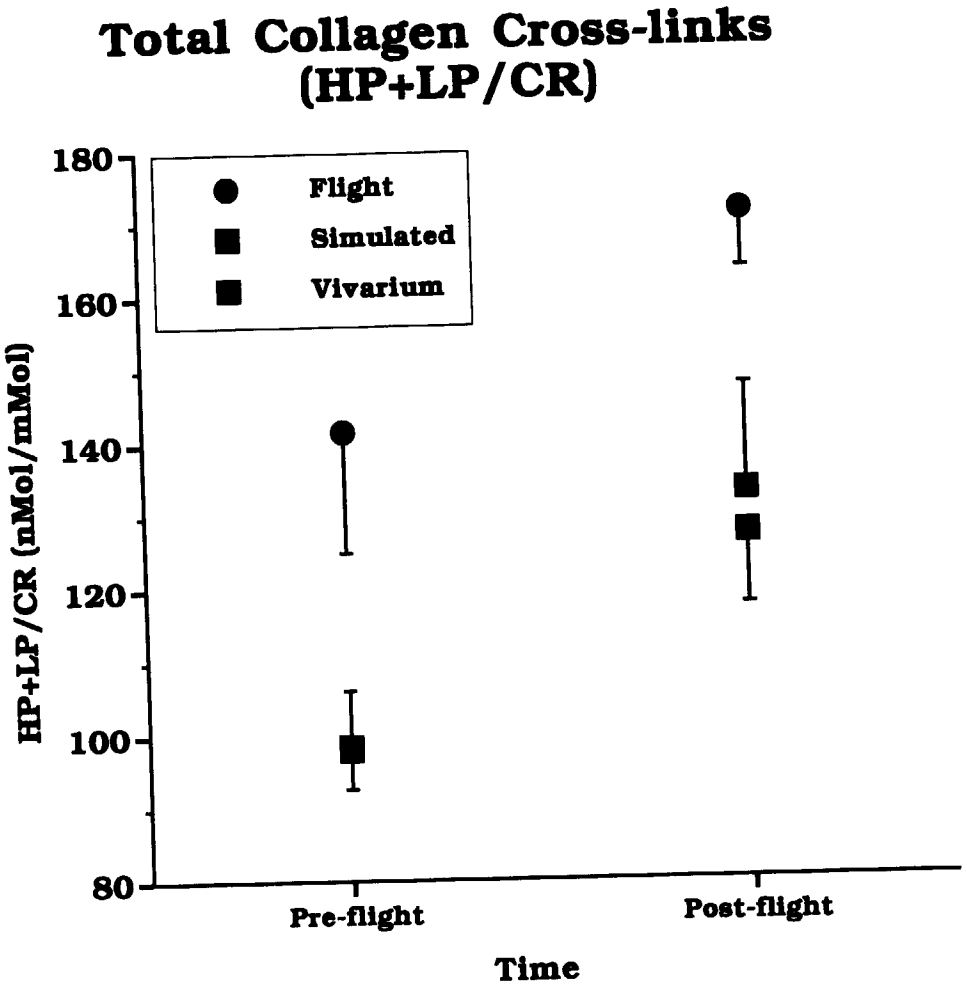
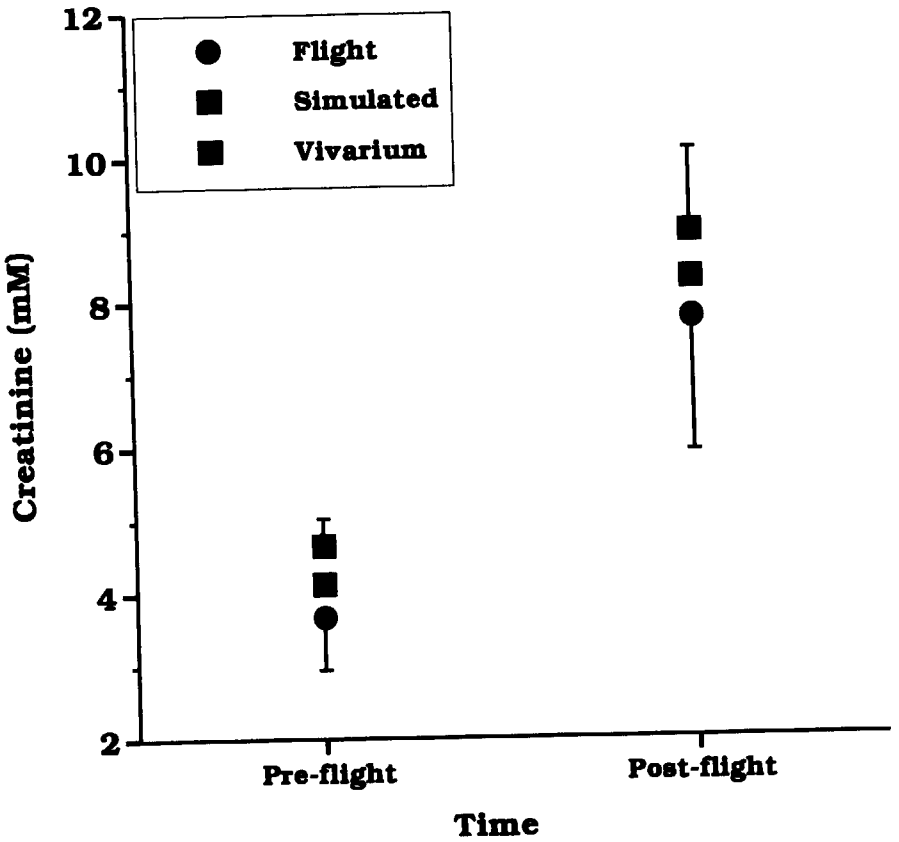
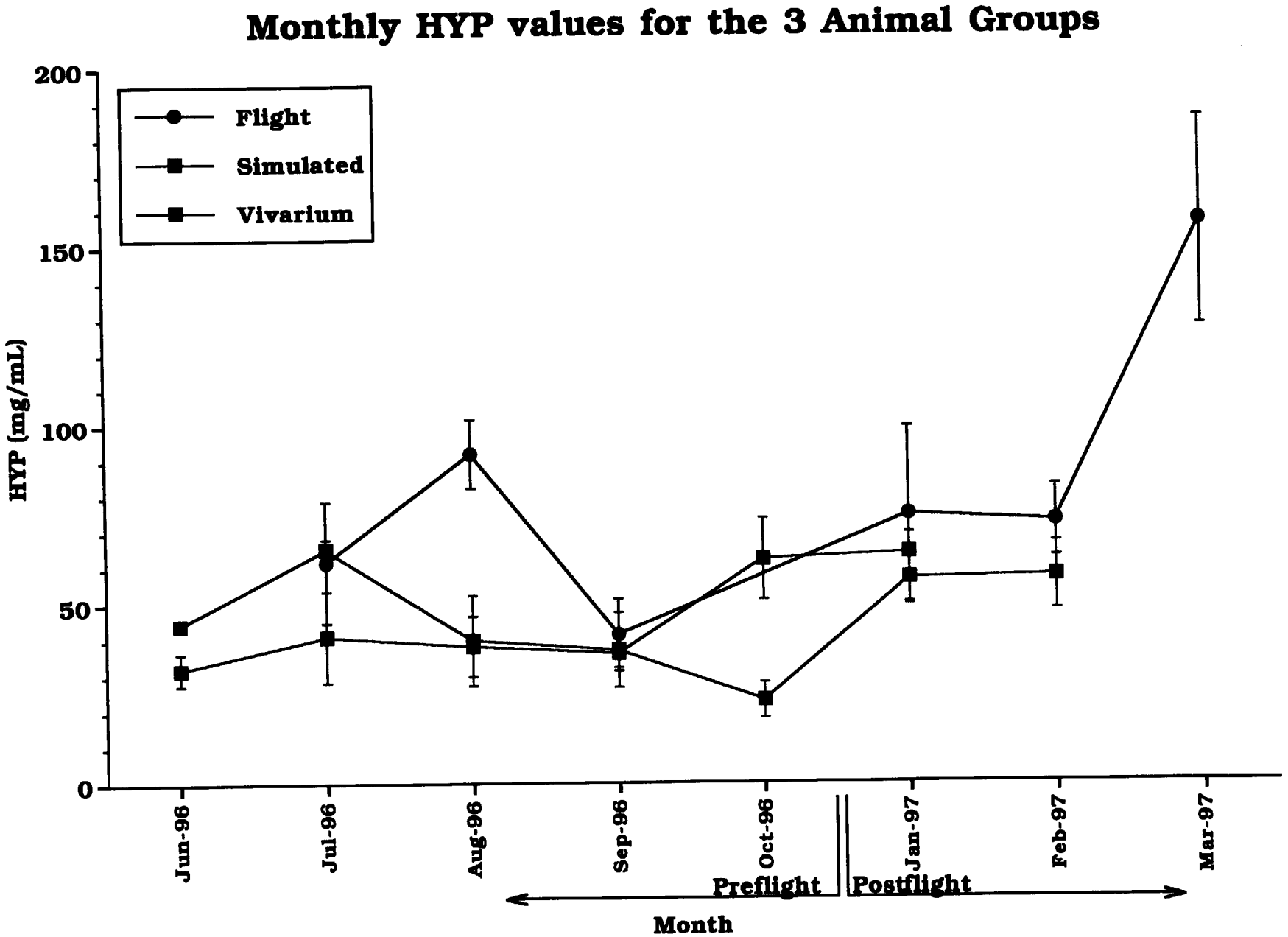
Flight
Simulated
Vivarium
LP (nM)
250
200
150
100
50
0
Pre-flight
Post-flight
Time

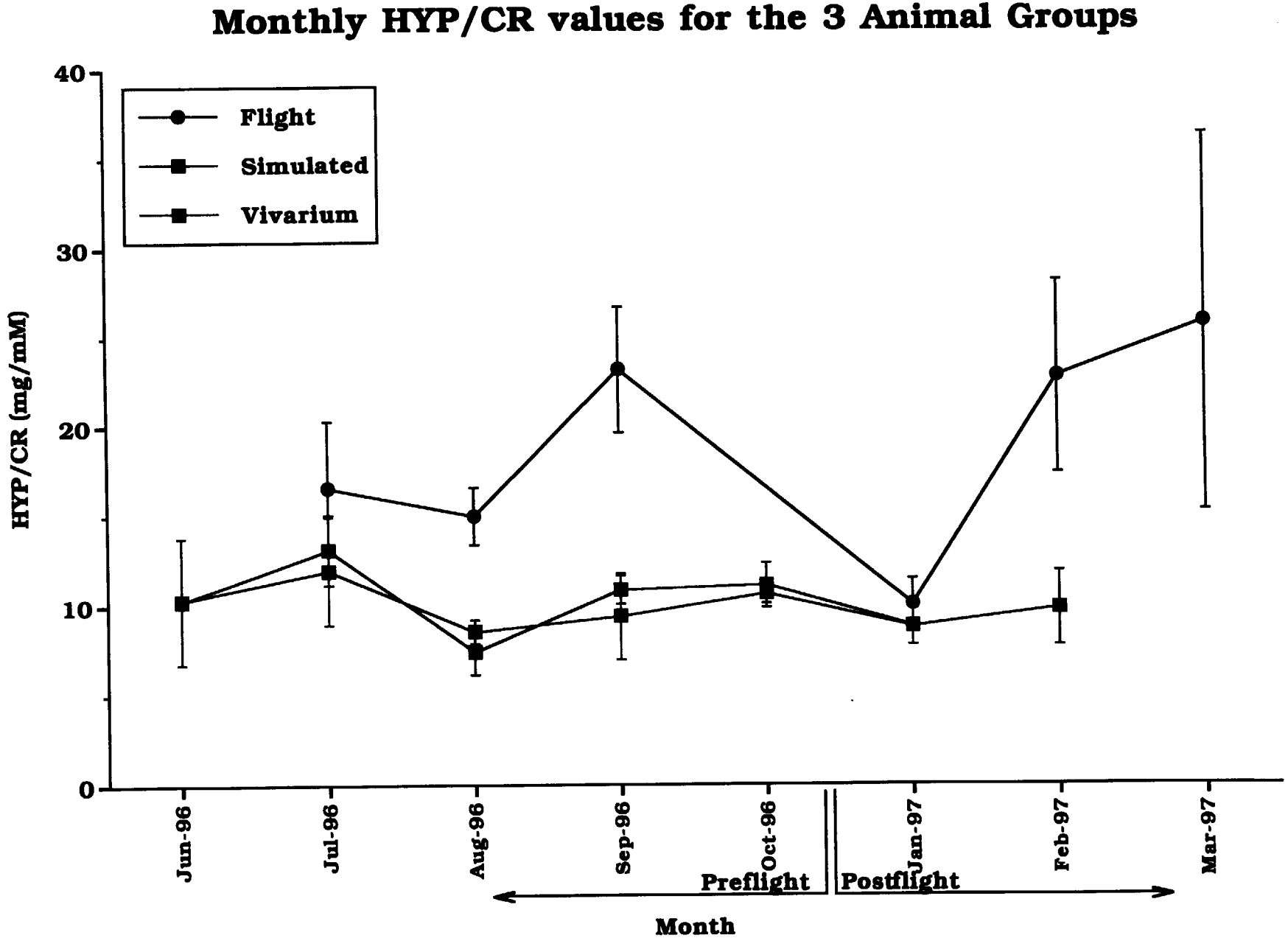

LP/CR values for the 3 Animal Groups
LP/CR (nMol/mMol)
30
25
20
15
10
5
0
Flight
Simulated
Vivarium
Pre-flight
Post-flight
Time

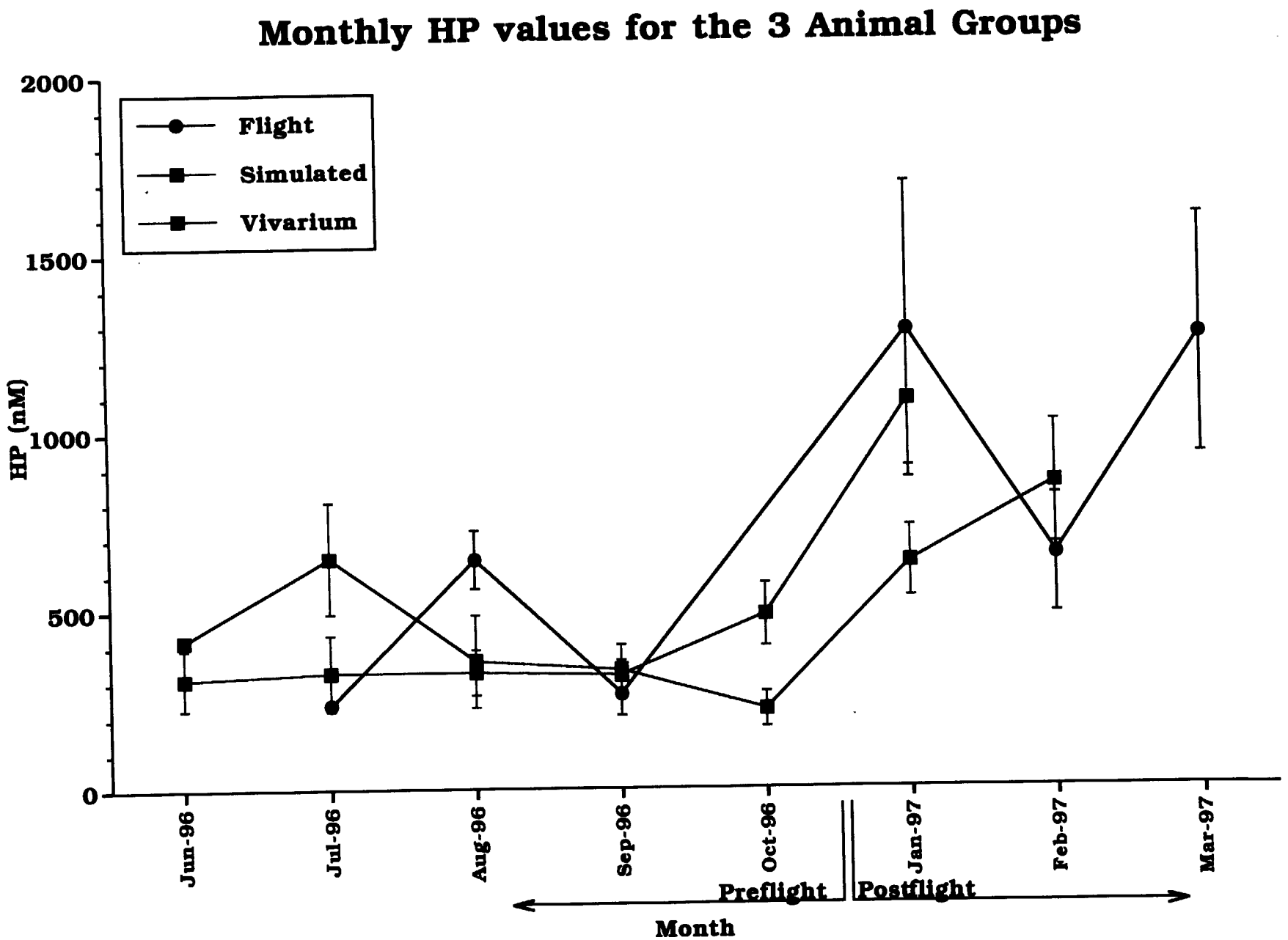

Total Collagen Cross-links
(HP+LP/CR)
Flight
Simulated
Vivarium
HP+LP/CR (nMol/mMol)
180
160
140
120
100
80
Pre-flight
Post-flight
Time

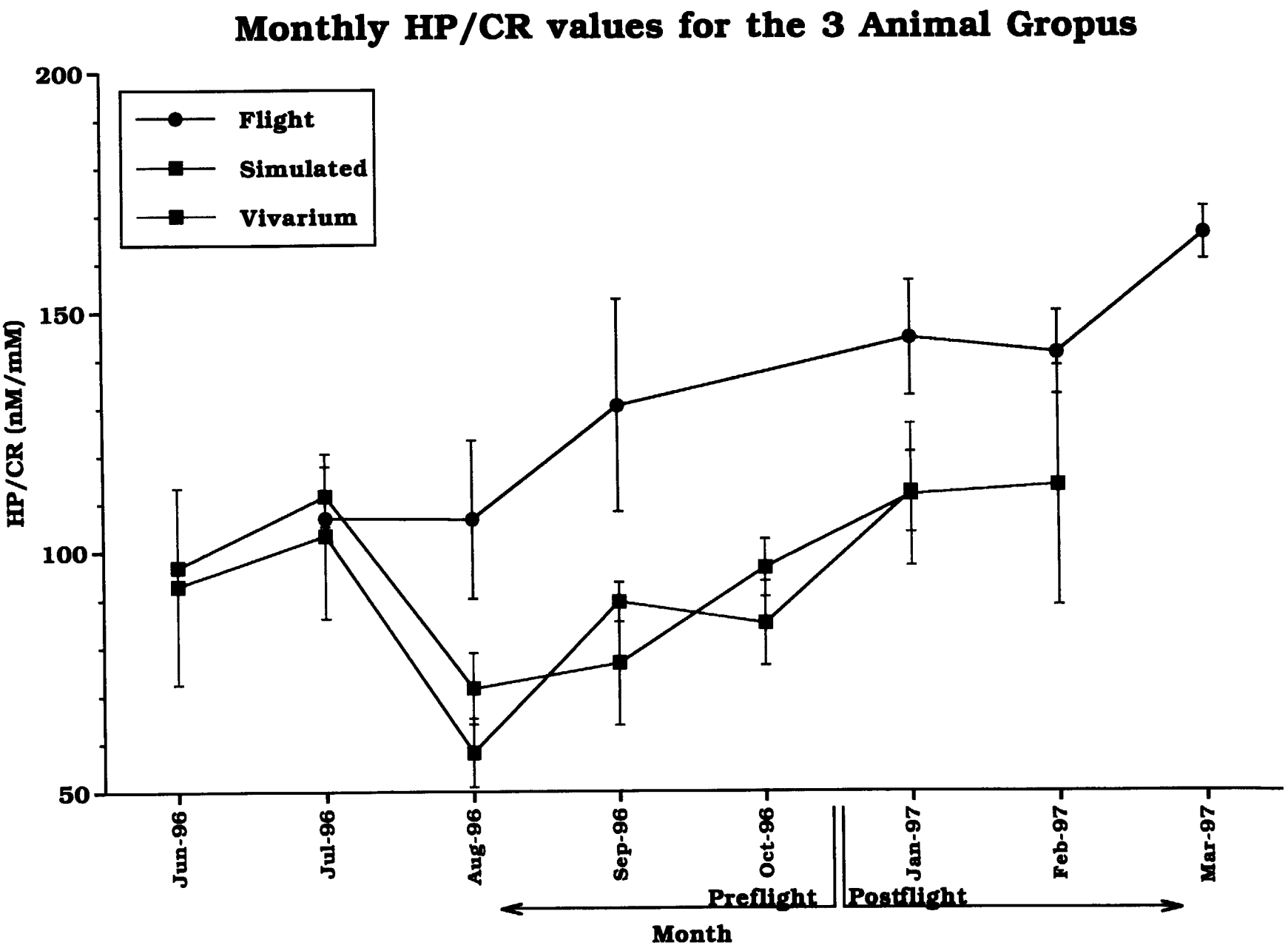
Creatinine values for the 3 animal groups




Monthly HYP values for the 3 Animal Groups
Flight
Simulated
Vivarium
HYP (mg/mL)
200
150
100
50
0
Jun-96
Jul-96
Aug-96
Sep-96
Oct-96
Jan-97
Feb-97
Mar-97
Preflight
Postflight
Month


Monthly HYP/CR values for the 3 Animal Groups
Flight
Simulated
Vivarium
HYP/CR (mg/mM)
40
30
20
10
0
Jun-96
Jul-96
Aug-96
Sep-96
Oct-96
Jan-97
Feb-97
Mar-97
Preflight
Postflight
Month


Monthly HP values for the 3 Animal Groups
Flight
Simulated
Vivarium
HP (nM)
2000
1500
1000
500
0
Jun-96
Jul-96
Aug-96
Sep-96
Oct-96
Jan-97
Feb-97
Mar-97
Preflight
Postflight
Month


Monthly HP/CR values for the 3 Animal Gropus
Flight
Simulated
Vivarium
HP/CR (nM/mM)
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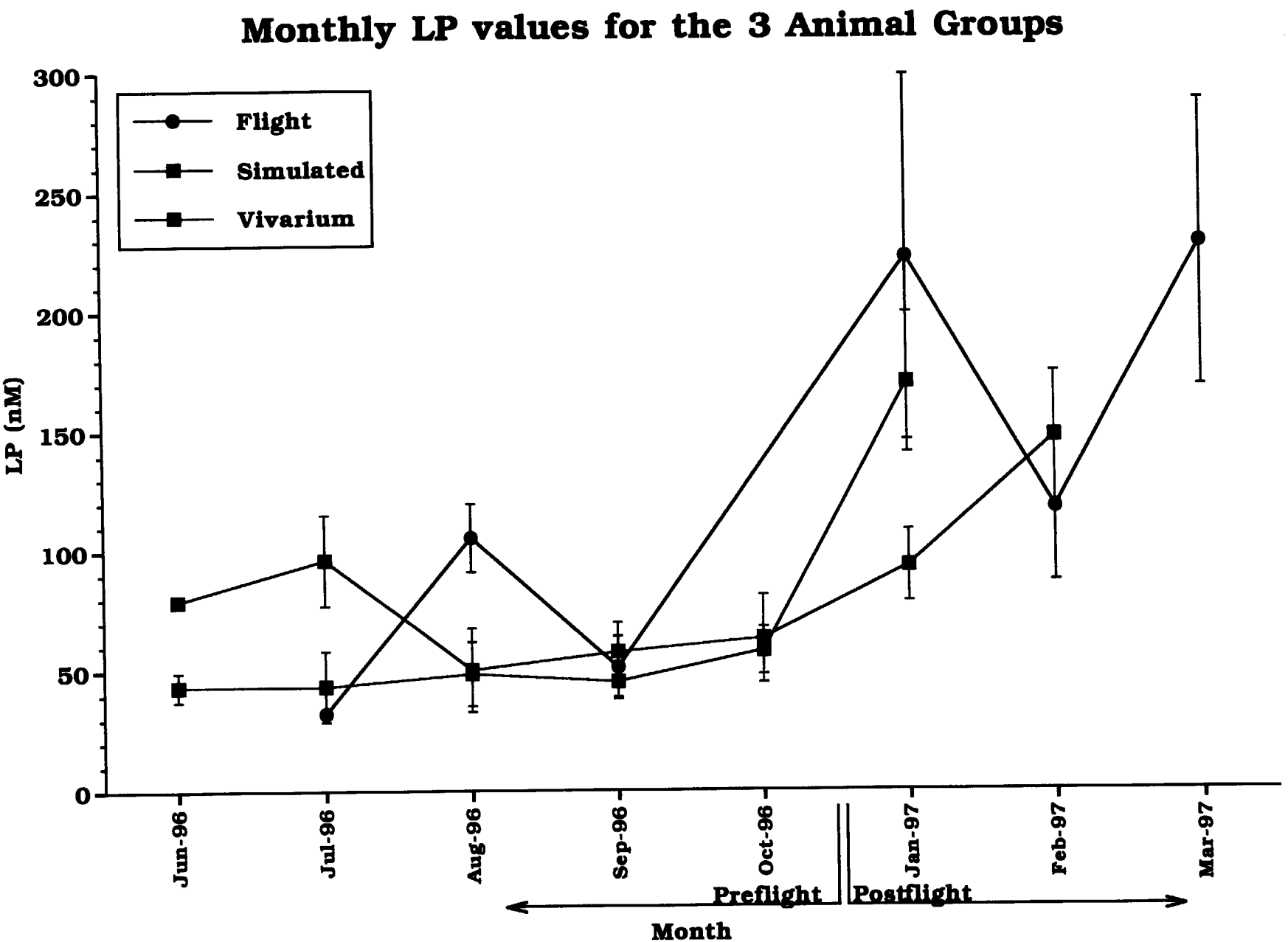
150
100
50
Jun-96
Jul-96
Aug-96
Sep-96
Oct-96
Jan-97
Feb-97
Mar-97
Preflight
Postflight
Month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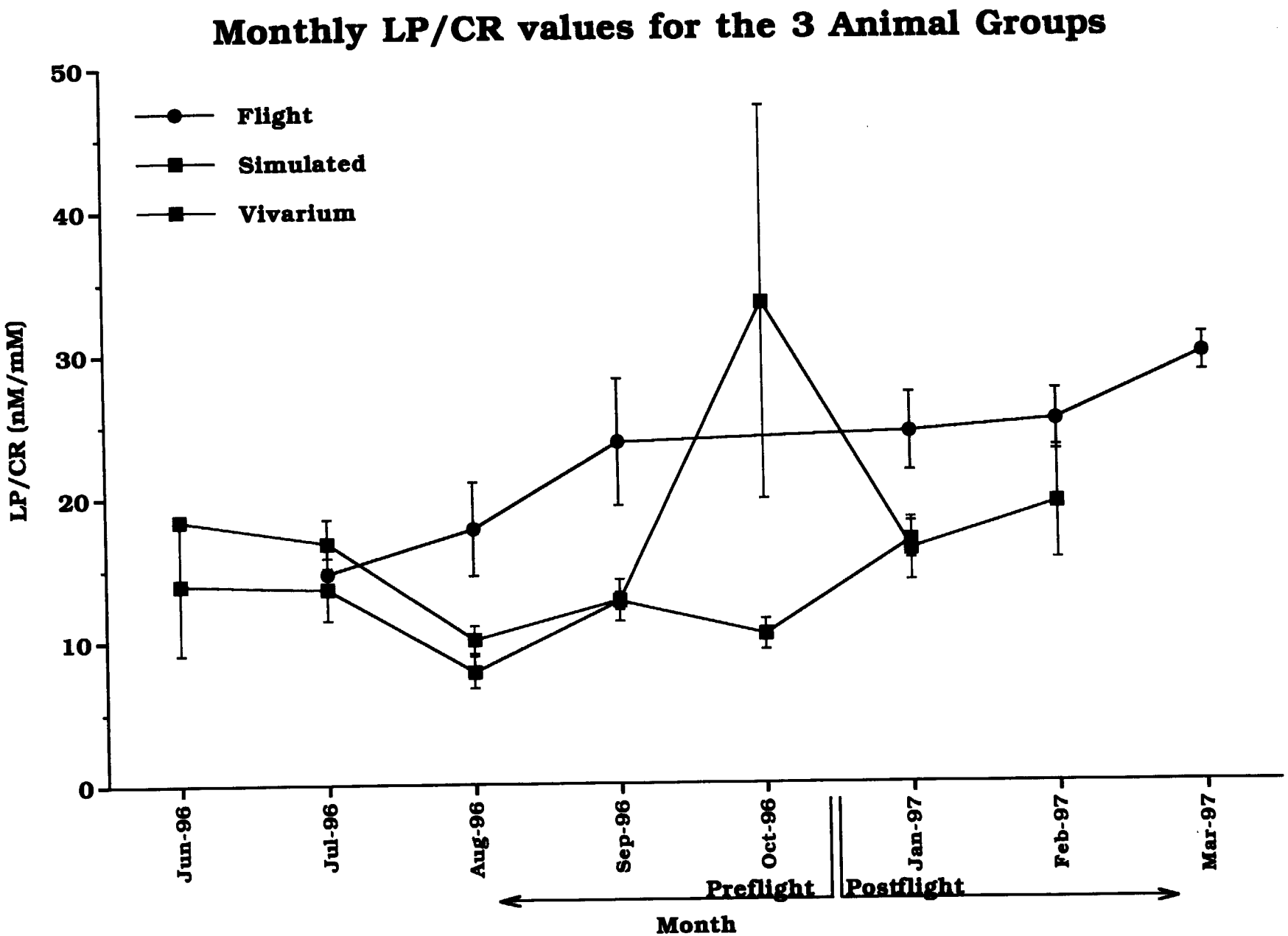
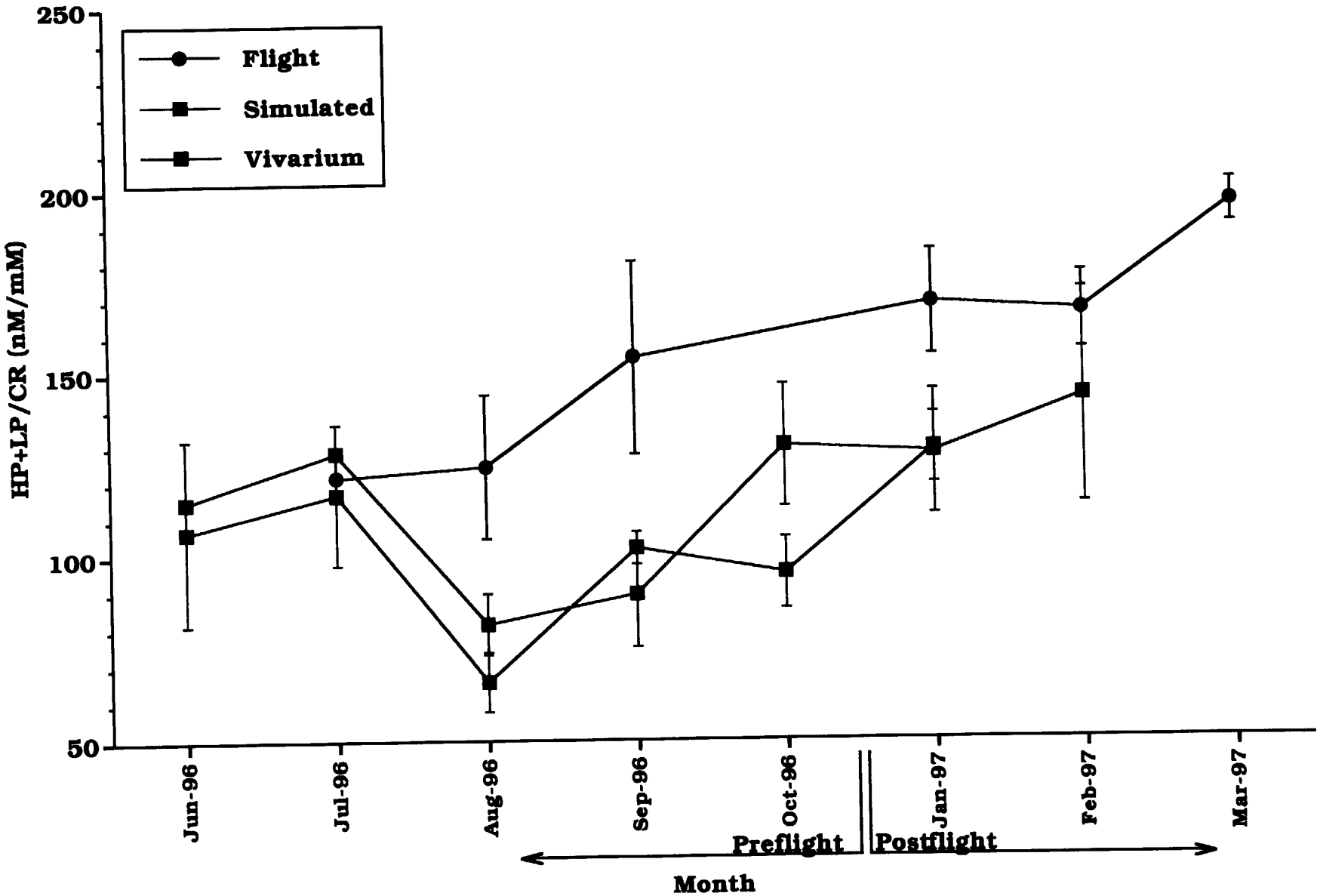
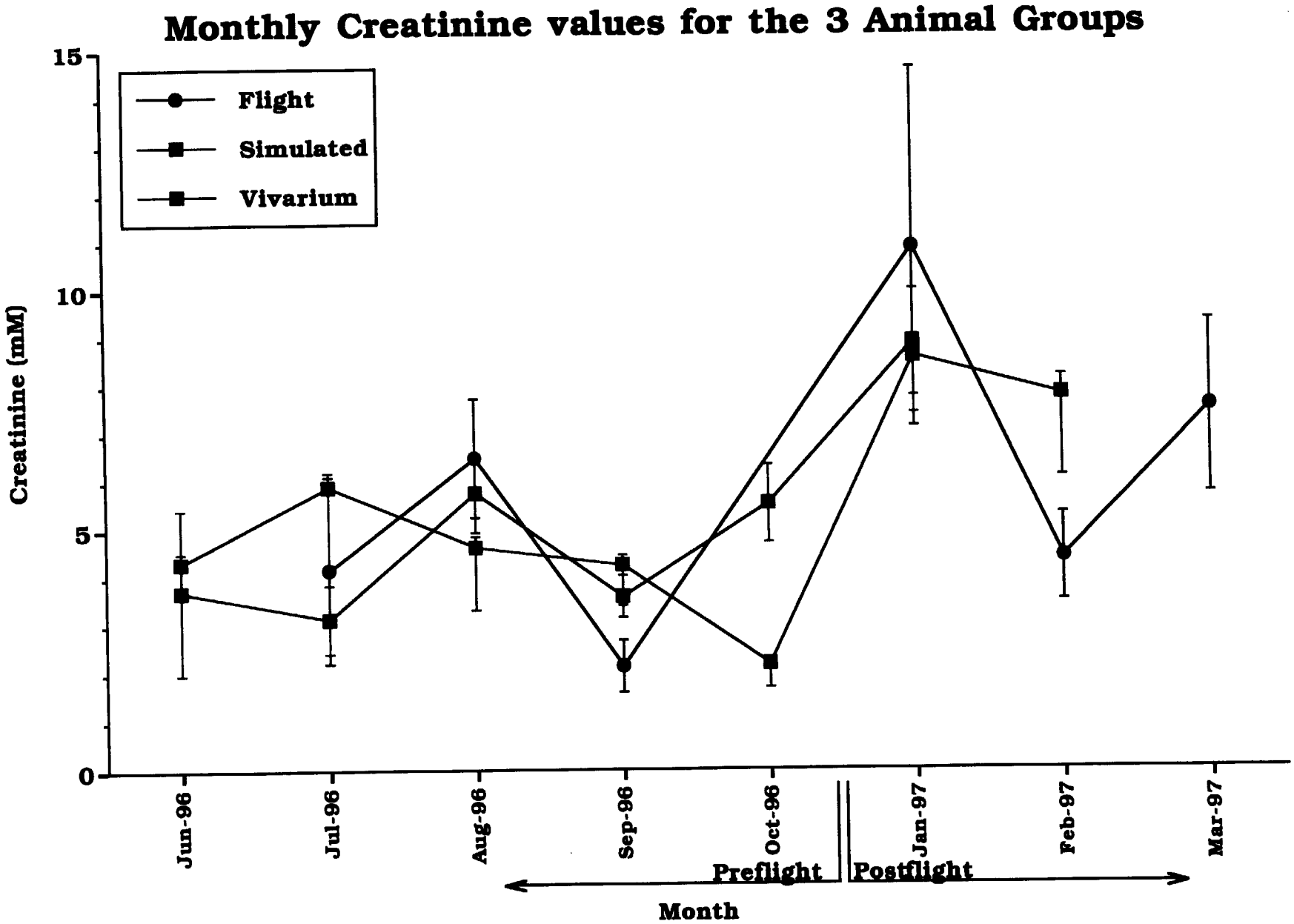
Flight
Simulated
Vivarium
LP (nM)
300
250
200
150
100
50
0
Jun-96
Jul-96
Aug-96
Sep-96
Oct-96
Jan-97
Feb-97
Mar-97
Preflight
Postflight
Month

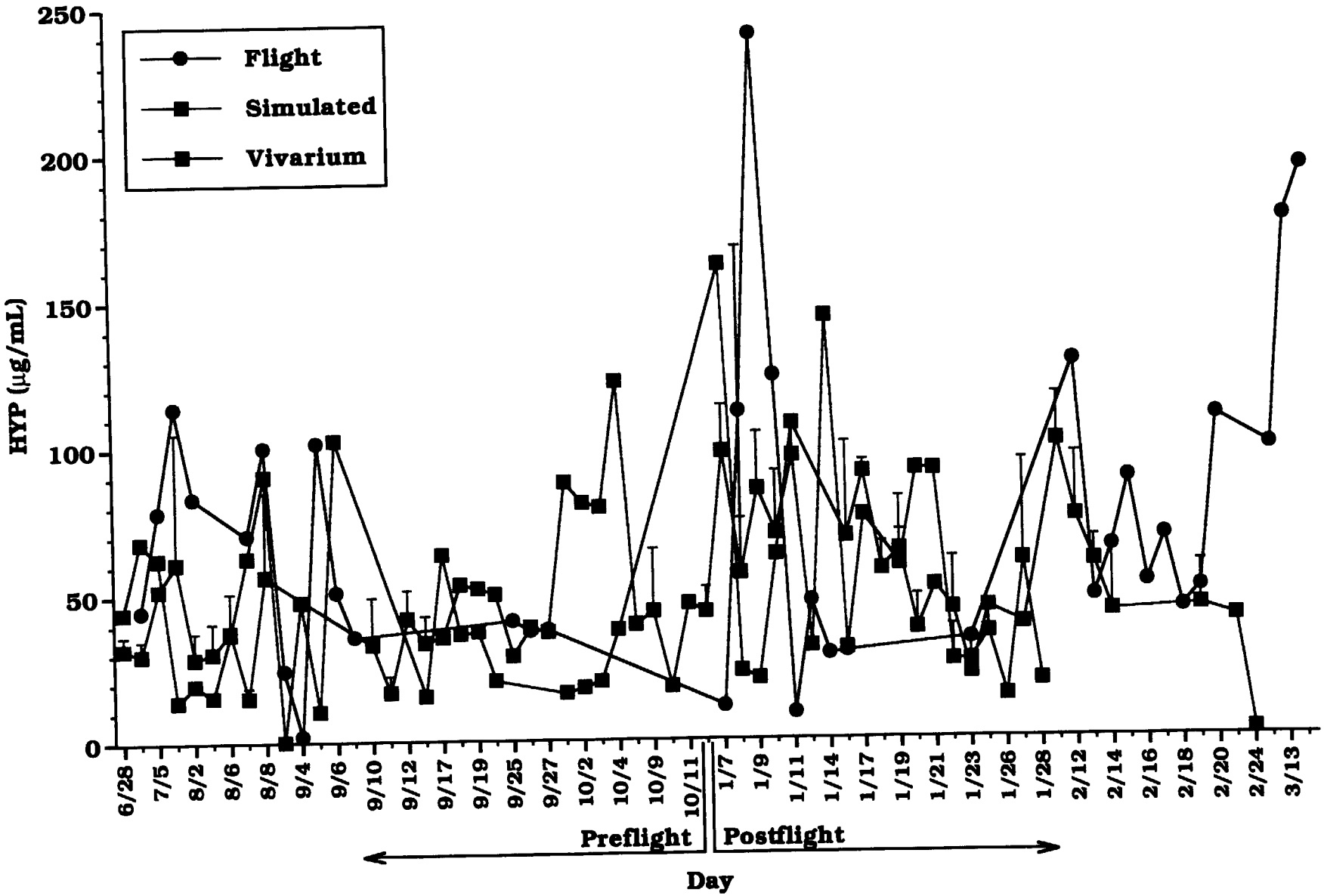

Monthly LP/CR values for the 3 Animal Groups
Flight
Simulated
Vivarium
LP/CR (nM/mM)
50
40
30
20
10
0
Jun-96
Jul-96
Aug-96
Sep-96
Oct-96
Jan-97
Feb-97
Mar-97
Preflight
Postflight
Month

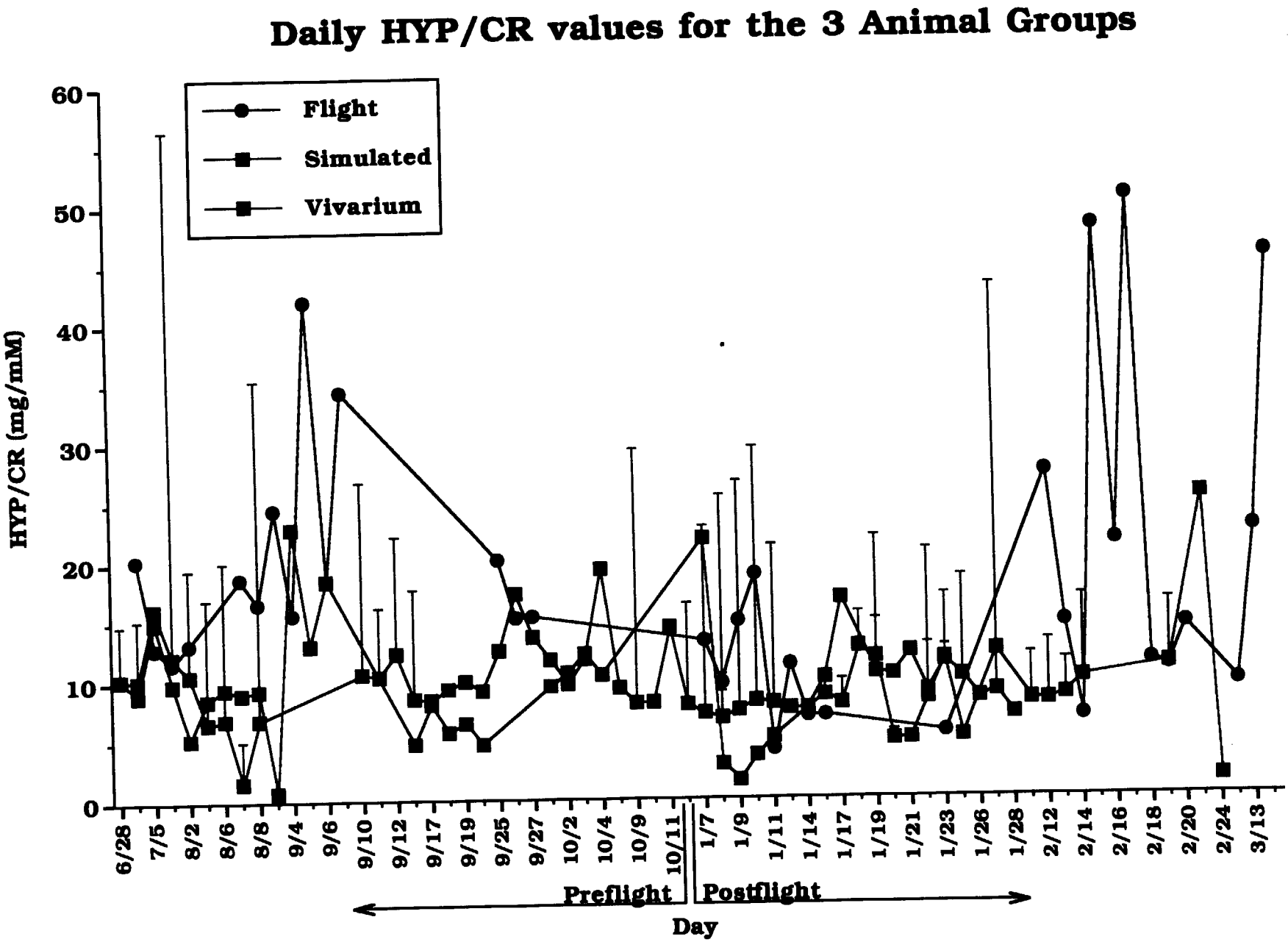
Monthly HP+LP/CR values for the 3 Animal Groups



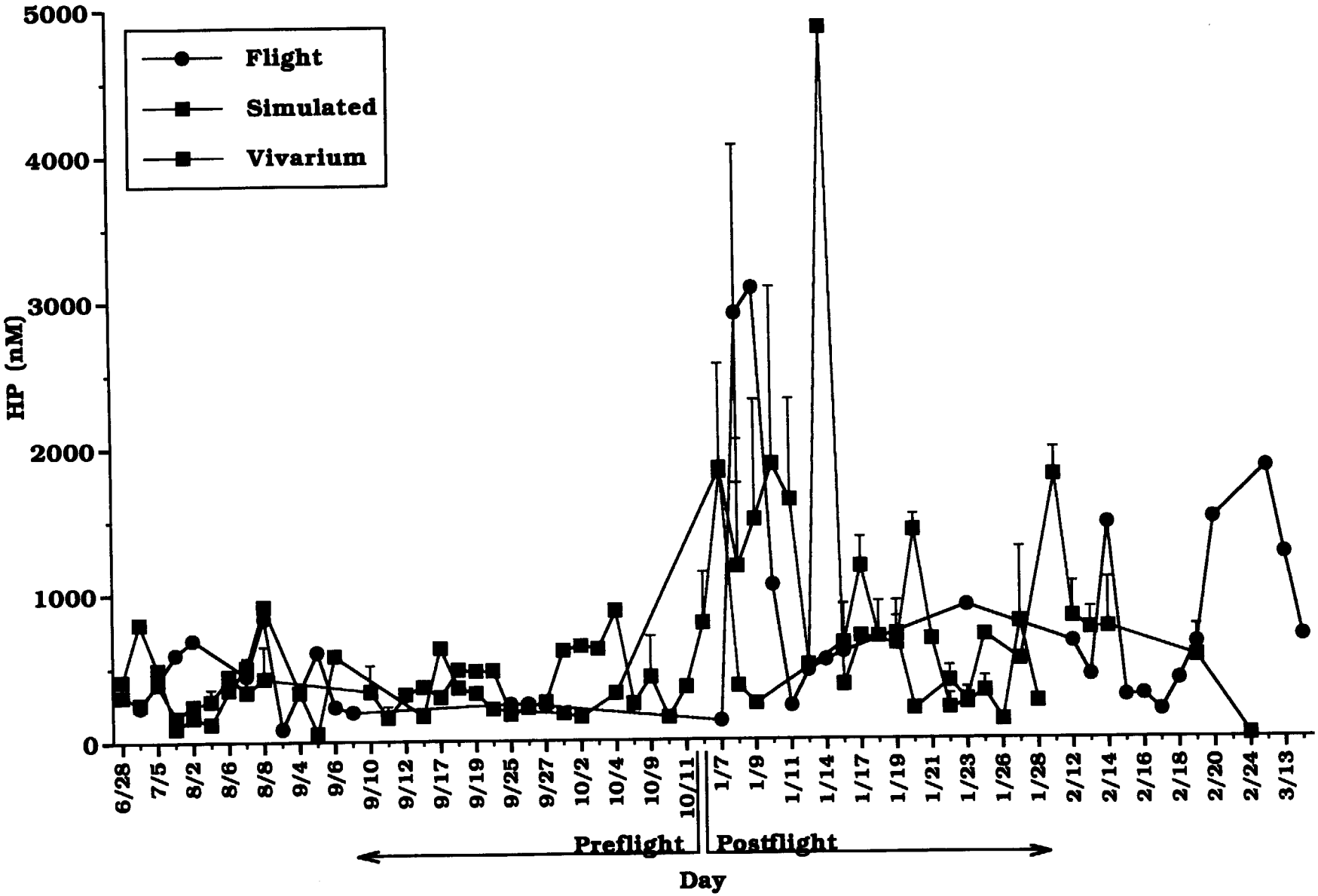

Monthly Creatinine values for the 3 Animal Groups
Flight
Simulated
Vivarium
Creatinine (mM)
15
10
5
0
Jun-96
Jul-96
Aug-96
Sep-96
Oct-96
Jan-97
Feb-97
Mar-97
Preflight
Postflight
Month

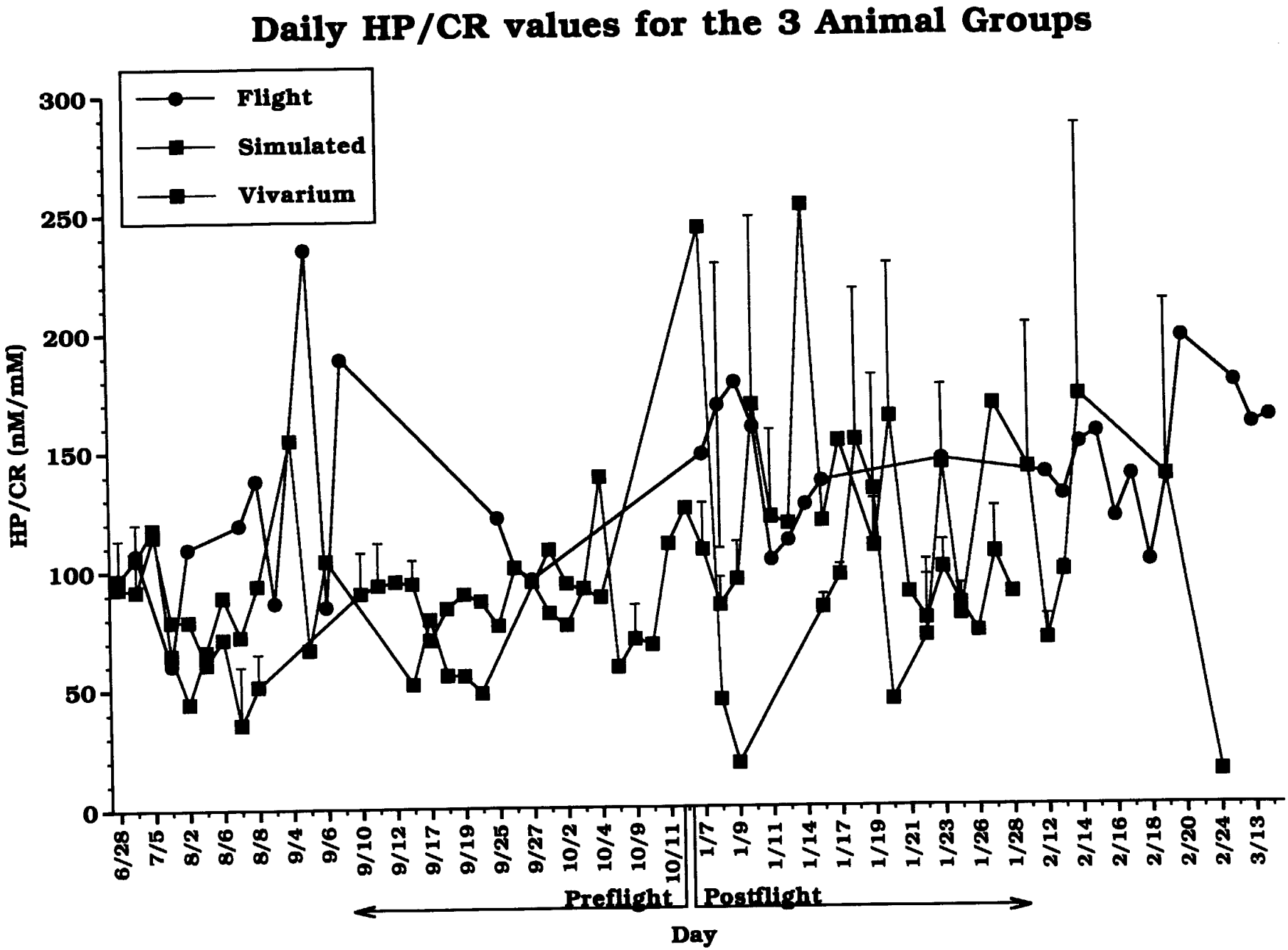
Daily HYP values for the 3 Animal Groups



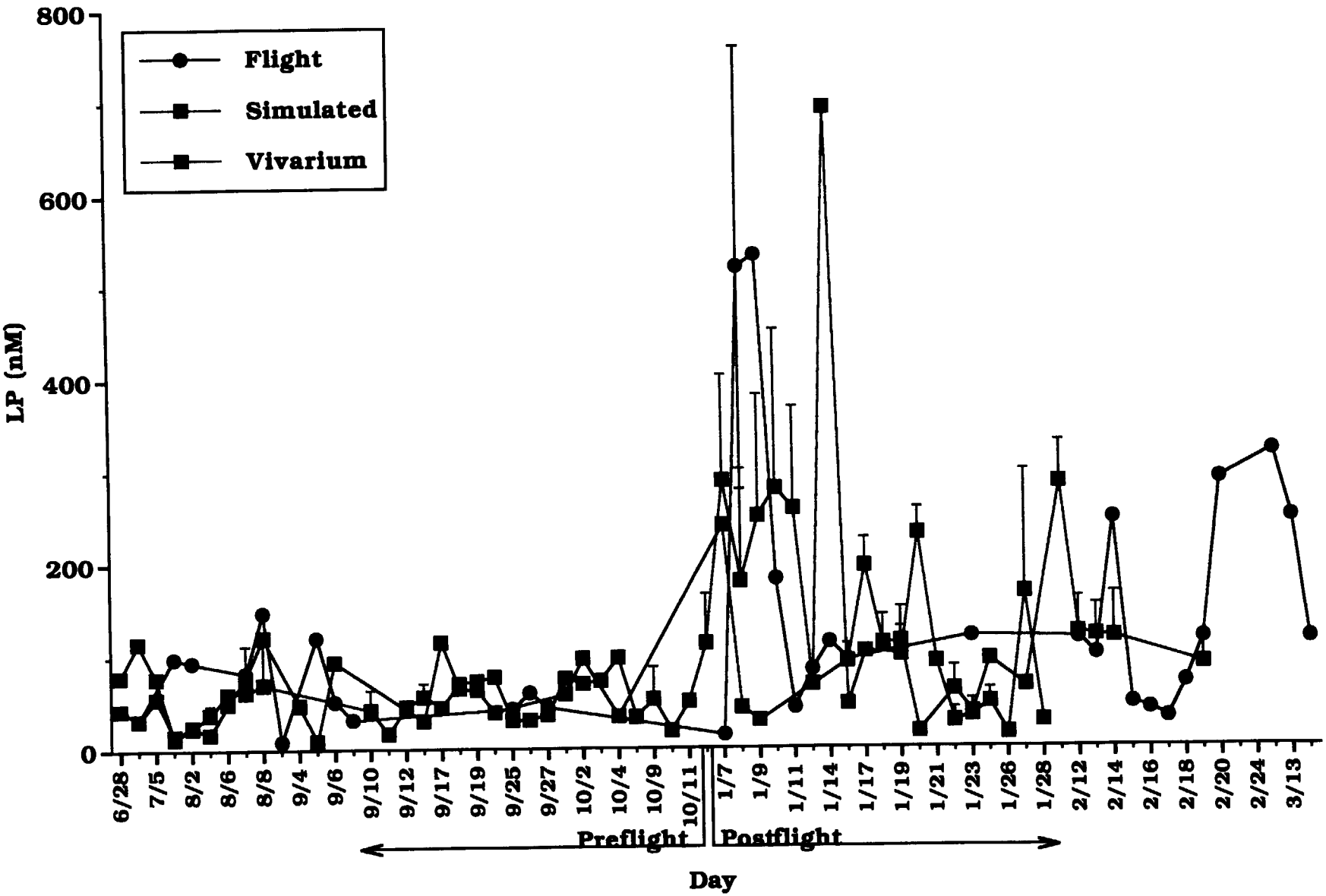

Daily HYP/CR values for the 3 Animal Groups
Flight
Simulated
Vivarium
HYP/CR (mg/mM)
60
50
40
30
20
10
0
6/28
7/5
8/2
8/6
8/8
9/4
9/6
9/10
9/12
9/17
9/19
9/25
9/27
10/2
10/4
10/9
10/11
1/7
1/9
1/11
1/14
1/17
1/19
1/21
1/23
1/26
1/28
2/12
2/14
2/16
2/18
2/20
2/24
3/13
Preflight
Postflight
Day

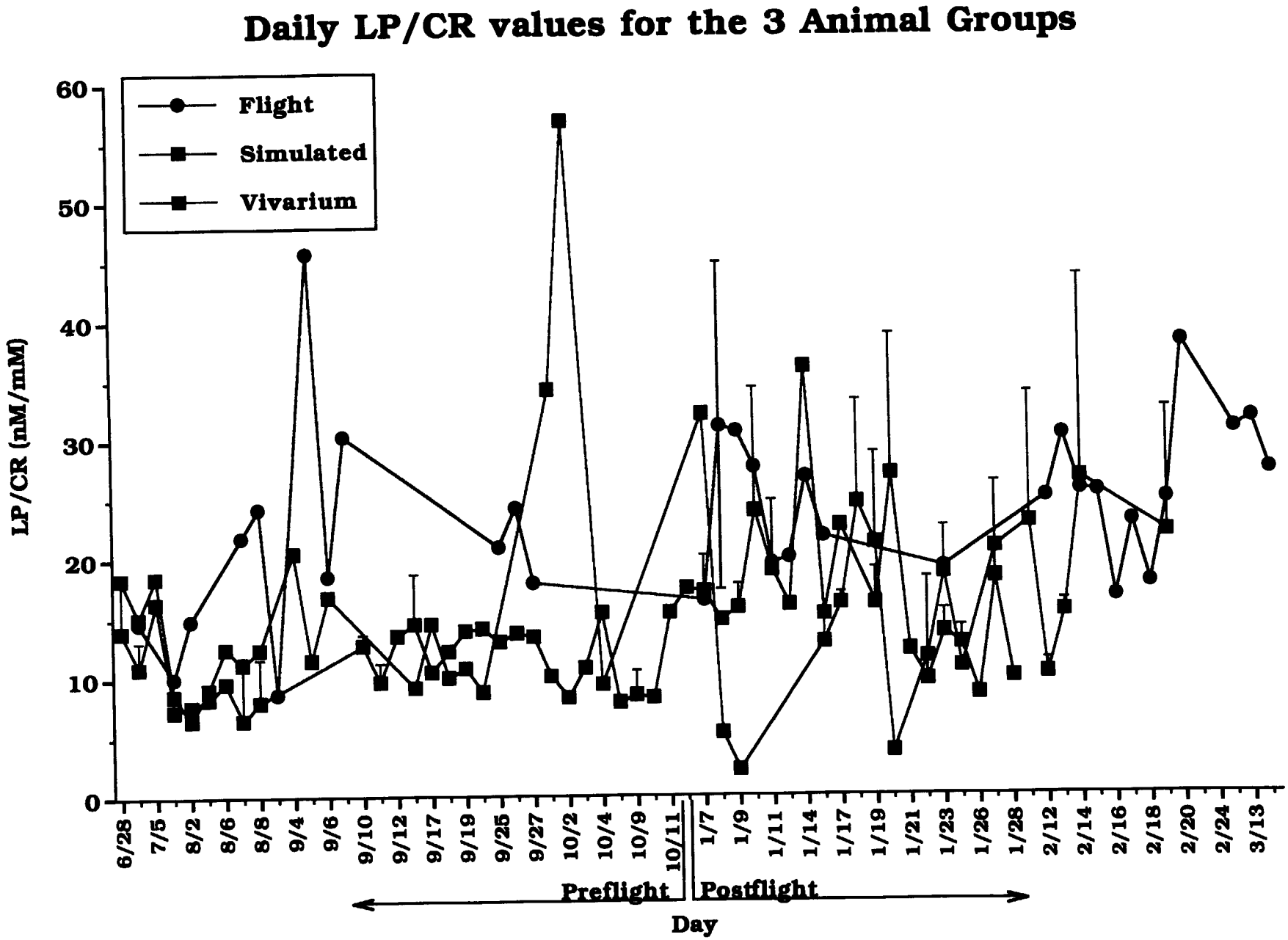
Daily HP values for the 3 Animal Groups



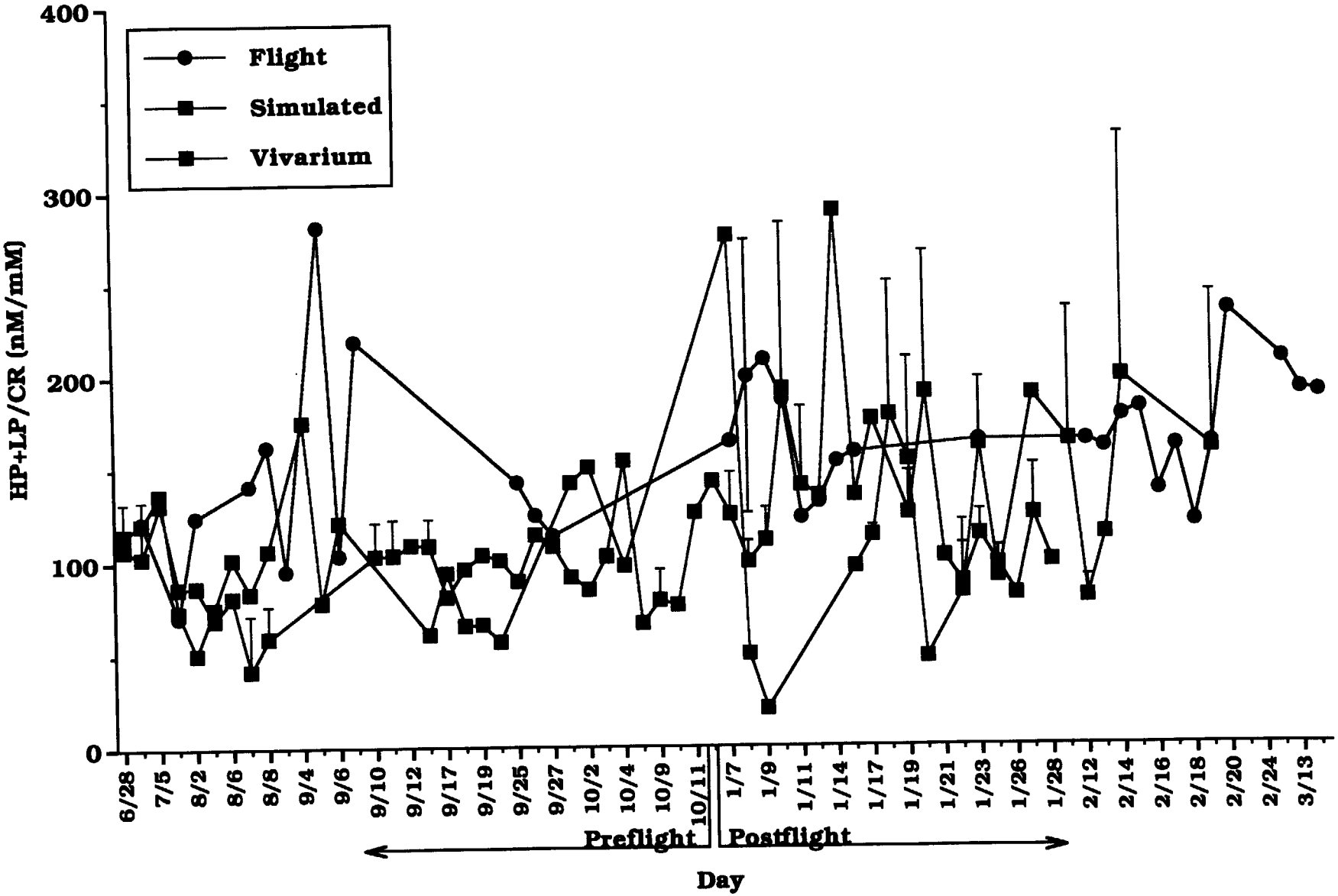

Daily HP/CR values for the 3 Animal Groups
Flight
Simulated
Vivarium
HP/CR (nM/mM)
300
250
200
150
100
50
0
6/28
7/5
8/2
8/6
8/8
9/4
9/6
9/10
9/12
9/17
9/19
9/25
9/27
10/2
10/4
10/9
10/11
1/7
1/9
1/11
1/14
1/17
1/19
1/21
1/23
1/26
1/28
2/12
2/14
2/16
2/18
2/20
2/24
3/13
Preflight
Postflight
Day

Daily LP values for the 3 Animal Groups




Daily LP/CR values for the 3 Animal Groups
Flight
Simulated
Vivarium
LP/CR (nM/mM)
60
50
40
30
20
10
0
6/28
7/5
8/2
8/6
8/8
9/4
9/6
9/10
9/12
9/17
9/19
9/25
9/27
10/2
10/4
10/9
10/11
1/7
1/9
1/11
1/14
1/17
1/19
1/21
1/23
1/26
1/28
2/12
2/14
2/16
2/18
2/20
2/24
3/13
Preflight
Postflight
Day

Daily HP+LP/CR values for the 3 Animal Groups



Daily Creatinine values for the 3 Animal Groups